Short Communication

Elucidation of the Role of Sugar Chains in Glycosylated-enzymes Using Endo-β-N-acetylglucosaminidase from Flavobacterium sp.

Kenji Yamamoto, Kaoru Takegawa, Hidehiko Kumagai and Tatsurokuro Tochikura

Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan

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Endo-β-N-acetylglucosaminidase (EC 3.2.1.96) hydrolyzes the glycosidic bonds between the core di-N-acetylchitobiose moieties of asparagine-linked oligosaccharides of various glycoproteins and releases the sugar moieties. This enzyme has been used for elucidation of the structures and functions of sugar moieties associated with glycoproteins. Recently, we found that a bacterial strain isolated from a soil sample produces a very high amount of endo-β-N-acetylglucosaminidase (endo-β-GlcNAcase) in the culture fluid. We identified the bacterium as Flavobacterium sp., and reported the purification and properties of the endo-β-GlcNAcase.1) Here we report that the carbohydrate moieties of glycoenzymes may protect them against proteolysis.

The term glycoenzyme has been used for enzymes that contain covalently linked carbohydrate residues in their structures.2) Yeast invertase, a representative glycoenzyme, contains a large amount of carbohydrates, about 50% of its molecular weight of about 270,000.3) Due to the attendant carbohydrates it has been difficult to measure the molecular weight of the enzyme accurately by various techniques.

The purified endo-β-GlcNAcase of Flavobacterium sp. removed the associated carbohydrate from yeast invertase.1) We investigated the differences between the native and the carbohydrate-depleted invertases. One unit of endo-β-GlcNAcase is defined as the amount of enzyme that yields 1 μmol of dansyl (5-dimethylaminonaphthalene-1-sulfonyl) asparagine-N-acetylglucosamine from dansyl ovalbumin glycopeptides as substrates per min at 37°C.1) Carbohydrate was removed from the native invertase by incubating 0.3 mg of the invertase with 0.5 units of the purified endo-β-GlcNAcase in a 0.1 ml reaction volume containing 100 mM acetate buffer, pH 6.0, which was confirmed by column (1.1 x 115 cm) chromatography on Sephadex G-100 and by 0.1% SDS-10% acrylamide slab gel electrophoresis (Fig. 1). SDS–electrophoresis of the native invertase gave a broad stained

Fig. 1. SDS–Polyacrylamide Gel Electrophoresis of Yeast Invertase.

Native or SDS-heat-treated invertase (0.3 mg) was incubated with 0.5 units of endo-β-GlcNAcase at 37°C for 18 hr in 100 mM acetate buffer, pH 6.0. Samples containing 60 μg of protein were electrophoresed on 10% polyacrylamide gel. Lanes (A) and (E) contained marker proteins. The other lanes contained: (B) native invertase, (C) endo-β-GlcNAcase-treated invertase, and (D) endo-β-GlcNAcase-treated SDS-heat-denatured invertase. Molecular weight markers were used are 1, phosphorylase b (MW 94,000); 2, bovine serum albumin (MW 67,000); 3, ovalbumin (MW 43,000); 4, carbonic anhydrase (MW, 30,000) and 5, soybean trypsin inhibitor (MW 20,100). The endo-β-GlcNAcase protein band was visualized, on Coomassie blue staining, at a position corresponding to a molecular weight of about 30,000.
band at a position corresponding to a molecular weight of about 120,000. After incubation with the endo-β-GlcNAcase, the carbohydrate-depleted invertase migrated as three stained bands to a position corresponding to about 65,000 daltons. The same result was obtained by Chu et al. using Streptomyces endo-β-GlcNAcase. According to their report, our data suggest that the endo-β-GlcNAcase of Flavobacterium sp. can liberate about 85% of the carbohydrate from the native invertase, and we verified this by obtaining the released carbohydrate chains by Sephadex G-100 gel filtration (although invertase activity was completely lost, all of the sugar chains seemed to be completely released by the endo-β-GlcNAcase when the native invertase was denatured by boiling in a water bath for 5 min in the presence of 0.25% SDS, as shown in Fig. 1). Invertase activity was assayed at 37°C by measuring the rate of reducing sugar release from sucrose according to the Nelson–Somogyi method. No differences were observed in enzymatic properties such as optimum pH, stable pH, and thermal stability between the native invertase and the carbohydrate-depleted invertase.

Then we investigated the inactivation by proteases of both the native invertase and the carbohydrate-depleted invertase. The native and carbohydrate-depleted forms of invertase (1 mg) were incubated with 0.1 mg of subtilisin (Sigma Chemical Co.) in 100 mM Tris–HCl buffer (pH 7.5), and both the invertase activities were assayed at various times. As shown in Fig. 2, the carbohydrate-depleted invertase was rapidly inactivated.

![Fig. 2. Effects of Protease Digestion on the Enzyme Activities of Native and Endo-β-GlcNAcase-treated Invertases.](image)

Native or endo-β-GlcNAcase-treated invertase (1 mg) was incubated with 0.1 mg of subtilisin at 37°C in 100 mM Tris–HCl buffer, pH 7.5. Samples were assayed for invertase activity by the Nelson–Somogyi method with sucrose as the substrate. (●), native invertase; (○), endo-β-GlcNAcase-treated invertase.

![Fig. 3. Degradation of Native and Carbohydrate-depleted Invertases by Subtilisin.](image)

Native or endo-β-GlcNAcase-treated invertase was incubated with subtilisin as described in the legend to Fig. 2. Samples were withdrawn for analysis by polyacrylamide gel electrophoresis. N. Inv., native invertase; Cd. Inv., carbohydrate-depleted invertase; Sub., subtilisin; Endo, endo-β-GlcNAcase; d. pep., degraded peptides from carbohydrate-depleted invertase by subtilisin.
In contrast, the native invertase seemed to be resistant to inactivation by the protease. The same result was obtained on the addition of pronase E (Kakenkagaku Co.) to both forms of invertase. Because of the above results, proteolytic degradation of invertases was analyzed by 0.1% SDS–10% acrylamide slab gel electrophoresis. As shown in Fig. 3, the native invertase was resistant to degradation, but the carbohydrate-depleted invertase was progressively converted to small peptides.

In conclusion, we were able to obtain a carbohydrate-depleted invertase by incubating the native form with endo-\( \beta \)-GlcNCase of Flavobacterium sp., but the enzyme was rapidly degraded and inactivated by protease. In contrast, the glycosylated form of this enzyme was significantly stable against proteolysis. Thus, a protective role of the carbohydrate in a glycoenzyme is suggested, using endo-\( \beta \)-GlcNCase of Flavobacterium sp. It is clear that this enzyme is an excellent tool for elucidation of the role of sugar moieties in glycoproteins.

REFERENCES